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Structure and Processivity of Two Forms of *Saccharomyces cerevisiae* DNA Polymerase δ*

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Yeast DNA polymerase δ (Polδ) consists of three subunits encoded by the *POL3*, *POL31,* and *POL32* genes. Each of these genes was cloned under control of the galactose-inducible *GAL1–10* promoter and overexpressed in various combinations. Overexpression of all three genes resulted in a 30-fold overproduction of Polδ, which was identical in enzymatic properties to Polδ isolated from a wild-type yeast strain. Whereas overproduction of *POL3* together with *POL32* did not lead to an identifiable Pol3p·Pol32p complex, a chromatographically distinct and novel complex was identified upon overproduction of *POL3* and *POL31*. This two-subunit complex, designated Polδ*, is structurally and functionally analogous to mammalian Polδ. The properties of Polδ* and Polδ were compared. A gel filtration analysis showed that Polδ* is a heterodimer (Pol3p·Pol31p) and Polδ a dimer of a heterotrimer, $(Pol3p·Pol31p·Pol32p)_2$. In the absence of proliferating cell nuclear antigen (PCNA), Polδ* showed a processivity of 2–3 on poly(dA)·oligo(dT) compared with 5–10 for Polδ. In the presence of PCNA, both enzymes were fully processive on this template. DNA replication by Polδ* on a natural DNA template was dependent on PCNA and on replication factor C. However, Polδ*-mediated DNA synthesis proceeded inefficiently and was characterized by frequent pause sites. Reconstitution of Polδ was achieved upon addition of Pol32p to Polδ*.

The subunit structure of eukaryotic DNA polymerase δ (Polδ)[1] remains ambiguous (for a review, see Ref. 1). The most thoroughly characterized form of mammalian Polδ is that isolated from calf thymus, a two-subunit enzyme with a 125-kDa catalytic subunit and a 48-kDa accessory subunit (2). The accessory subunit is required for efficient stimulation of Polδ by the proliferating cell nuclear antigen (PCNA) (3, 4). Likewise, mouse Polδ can be purified in two forms, the single catalytic subunit form which is not stimulated by PCNA and the two-subunit enzyme which is stimulated by PCNA (5). The subunit composition of the two-subunit enzymes is that of a heterodimer (2, 5). In contrast, Polδ isolated from the two yeasts is more complex with the enzyme from *Saccharomyces cerevisiae* having three subunits and that from *Sshizosaccharomyces pombe* at least four, and perhaps five subunits (6, 7).

The three subunits of *S. cerevisiae* Polδ have apparent sizes by SDS-PAGE of 125, 58, and 55 kDa and are encoded by the *POL3*, *POL31,* and *POL32* genes, respectively (7). The 125-kDa catalytic subunit encoded by the *POL3* (*CDC2*) gene is very highly conserved in all eukaryotes (1). The 58-kDa second subunit is encoded by the *POL31* (*HYS2, SDP5*) gene (7). Mutations in this essential gene can cause sensitivity to the replication inhibitor hydroxyurea, for the *hys2-1* allele, or suppress the temperature sensitivity of mutations in the catalytic subunit, for the *sdp5-1* allele (8, 9). Pol31p shows 23–28% sequence similarity to the 48-kDa subunit of human Polδ and to *S. pombe* Cdc1. The essential $cdc1^+$ gene encodes the second subunit of *S. pombe* Polδ (6). The 55-kDa subunit is encoded by the *POL32* gene. Mutants deleted for *POL32* are viable, but show both replication and repair defects (7). Although the sequence similarity between Pol32p and Cdc27, which is the essential third subunit of *S. pombe* Polδ, is very low, other considerations including the presence of a PCNA-binding motif in both subunits denote these two as functional homologues (discussed in Ref. 7).

In the previous paper (7) we have described the cloning of the two small subunit genes of Polδ and their characterization. Here we describe the overproduction in yeast of the three-subunit form of Polδ, and of a two-subunit form, called Polδ*, which is analogous to mammalian Polδ.

MATERIALS AND METHODS

Strains and Plasmids

The yeast strains used in this work are the protease-deficient galactose-inducible strains BJ2168 (*MAT*a, *ura3-52*, *trp1-289*, *leu2-3, 112*, *prb1–1122*, *prc1-407*, *pep4-3*), PY116 (*MAT***a** *ura3-52 trp1-Δ his3-11, 15 leu2-3, 112 pep4-3 prb1–1122 nuc1::LEU2*) and its *pol32Δ* derivative PY117 (*MAT***a** *ura3-52 trp1-Δ his3-11, 15 leu2-3, 112 pep4-3 prb1–1122 nuc1::LEU2 pol32Δ::HIS3*) (7).

The overproduction plasmids used in this study are based upon the pRS420 series plasmids into which the *GAL1–10* upstream activating sequence (GAL1–10 upstream activating sequence) including the transcriptional start sites for the *GAL1* and *GAL10* genes, as a 678-nt *Bam*HI-*Eco*RI fragment, was inserted into the corresponding plasmid polylinker sites, resulting into vectors pRS424-GAL (*TRP1*), pRS425-GAL (*LEU2*), and pRS426-GAL (*URA3*) (10). All vectors have in addition the yeast 2 μM origin for high copy maintenance in yeast and the Bluescript $SKII^+$ backbone for propagation in *E. coli*. The transcriptional start site of the *GAL1* gene is 60 nt upstream of the *Bam*HI cloning site and the transcriptional start site of the *GAL10* gene is 10 nt upstream of the *Eco*RI cloning site. Both promoters are of similar strength. Coordinates are with reference to the translational start sites. pBL336 (*TRP1 GAL1-POL3*) has a 3.6-kb *Hgi*AI (trimmed)-*Hin*dIII fragment (coordinates: −45 to 3543) cloned into the *Bam*HI (filled)-*Hin*dIII sites of pRS424-GAL. pBL338 (*LEU2 GAL1-POL31*) has a 1.6-kb *Nco*I (filled)-*Cla*I (filled) fragment (coordinates: 2 to 1567) from pBL361 cloned into the *Sac*II site of pRS425-GAL (7). pBL340 (*URA3 GAL10-POL32*) has a 1.7-kb *Hpa*I-*Sal*I fragment (coordinates: −20 to 1688) from pBL384 cloned into the *Eco*RI (filled)-*Sal*I sites of pRS426-GAL (7).

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[1] The abbreviations used are: Polδ, DNA polymerase δ; Polδ*, Polδ lacking Pol32p; SS, single-stranded; PCNA, proliferating cell nuclear antigen; RFC, DNA replication factor C; DTT, dithiothreitol; BuPh-dGTP, N^2-(*p-n*-butylphenyl)-2′-deoxyguanosine-5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; nt, nucleotide; kb, kilobase(s).

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Cell Growth

A single colony of a plasmid-containing strain from a selective SCGL plate was grown in an air shaker at 30 °C in 100 ml of selective SCGL medium. SCGL medium contains per liter: 1.7 g of yeast nitrogen base without amino acids and ammonium sulfate, 5 g of ammonium sulfate, 30 ml of glycerol, 20 ml of lactic acid, 1 g of glucose, 20 g of agar for solid media, 20 mg each of adenine, uracil, histidine, tryptophan, proline, arginine, and methionine, 30 mg each of isoleucine, tyrosine, and lysine, 50 mg of phenylalanine, and 100 mg each of leucine, glutamic acid, aspartic acid, valine, threonine, and serine. Uracil, tryptophan, and/or leucine were omitted when appropriate to ensure the selective maintenance of plasmids. Prior to autoclaving, the pH of the media was adjusted to 5–6 with concentrated sodium hydroxide. After 2–3 days when the OD_{660} had reached 0.8–1, the culture was used to inoculate 1200 ml of SCGL media. After overnight growth, when the OD_{660} was about 1, 1200 ml of YPGL were added. YPGL contains per liter: 10 g of yeast extract, 20 g of peptone, 30 ml of glycerol, 20 ml of lactic acid, 2 g of glucose, and 20 mg of adenine. Prior to autoclaving, the pH of the media was adjusted to 5–6 with concentrated sodium hydroxide. The culture was equally divided over two 4-liter flasks and grown at 30 °C for 3 h. Solid galactose (2% final concentration) was then added to each flask and after 4 h of continuous shaking the cells were harvested.

Enzyme Purification

All steps were carried out at 0–4 °C. The following buffers were used: buffer A: 0.1 M Tris-HCl, pH 7.8, 5% (v/v) glycerol, 175 mM ammonium sulfate, 2 mM EDTA, 1 mM EGTA, 3 mM DTT, 0.025% Nonidet P-40, 5 μM pepstatin A, 5 μM leupeptin, 2 μg/ml chymostatin, 0.5 mM *p*-methylphenylsulfonyl fluoride, 5 mM benzamidine,10 mM $NaHSO_3$. Buffer B consisted of 25 mM KH_2PO_4, pH 7.5, 10% glycerol, 2 mM EDTA, 1 mM EGTA, 3 mM DTT, 0.01% Nonidet P-40, 5 μM pepstatin A, 5 μM leupeptin, 2 μg/ml chymostatin, 0.5 mM *p*-phenylsulfonyl fluoride. Buffer C was 30 mM triethanolamine-HCl, pH 7.3, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 0.01% Nonidet P-40, 3 mM DTT, 5 μM pepstatin A, 5 μM leupeptin, 5 mM $NaHSO_3$. Buffer D was 30 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, 0.01% Nonidet P-40, 5 mM DTT, 2 μM pepstatin A, 2 μM leupeptin, 0.1% (v/v) ampholytes 3.5–9. Salt concentrations (as NaCl) are indicated by a suffix, *e.g.* Buffer A_{500} = Buffer A + 500 mM NaCl. Buffers were precooled on ice water.

Bead beating was carried out in a 350-ml chamber containing 175 ml of glass beads (0.4–0.5 mm diameter) and 80–100 g, wet weight, of cells, resuspended in an equal volume of 2 × buffer A. The chamber was cooled in ice water and the beater turned on for 45 s, followed by a cooling period of 2 min, for a total beating time of 5 min. The lysate was poured in a cold graduated cylinder and the beads were washed with 50 ml of extraction buffer. An aliquot was centrifuged at 45,000 × *g* for 20 min (cleared lysate, see Table I). The volume of the crude lysate was measured and 40 μl of 10% Polymin P were added per ml of lysate. After 5 min of mixing, the lysate was spun for 40 min at 13,000 rpm in a GSA rotor. Solid ammonium sulfate (0.28 g/ml) was added to the supernatant and dissolved by stirring. The precipitate was collected at 13,000 rpm for 45 min. The pellet was resuspended in 20 ml of Buffer B. After dialysis against 2 × 500 ml of buffer B for 8 h each, the dialysate was cleared by centrifugation at 18,000 rpm for 20 min.

The cleared ammonium sulfate fraction was loaded on a 20-ml phosphocellulose column, equilibrated in buffer B. The column was washed with 40 ml of B_{25} and eluted with B_{750}. The protein-containing fractions were combined and dialyzed for 2 × 3 h against 150 ml each of buffer C until the conductivity of the dialysate was equal to that of C_{25}.

The dialyzed fraction was injected onto a 8-ml MonoQ column, equilibrated in buffer C_{25}, washed with 10 ml of C_{25}, and eluted with a 120-ml linear gradient from C_{25} to C_{500}. Fractions of 2.5 ml were collected, Polδ* eluted at $\sim C_{150}$ and Polδ at $\sim C_{200}$.

Individual Mono Q fractions were diluted with 2 volumes of buffer D and injected onto a 1-ml MonoS column, equilibrated in buffer D_{50}. The column was washed with 2 ml of D_{50} and eluted with a 15-ml linear gradient from D_{50} to D_{500}. Polδ* eluted at $\sim D_{250}$ and Polδ at $\sim D_{400}$.

Samples of 200 μl were injected onto a 20-ml Superose 6 column in 40 mM Hepes-NaOH, pH 7.5, 10% ethylene glycol, 1 mM EDTA, 0.02% Nonidet P-40, 0.2 M NaCl, 5 mM DTT, 5 mM $NaHSO_3$, and 2 μM each of leupeptin and pepstatin A. The column was run at 0 °C at 0.2 ml/min. Fractions of 300 μl were collected and analyzed by 10% SDS-PAGE and for DNA polymerase activity.

Enzyme Assays

DNA Polymerase—The DNA polymerase assay on activated DNA is described in the previous paper (7).

Stimulation of Polδ by PCNA on Poly(dA)-Oligo(dT)—The 10-μl reaction contained 20 mM Tris-HCl, pH 7.8, 8 mM $MgAc_2$, 0.2 mg/ml bovine serum albumin, 1 mM dithiothreitol, 25 μM [α-^{32}P]dTTP, 100 ng of poly(dA)-$(dT)_{22}$ (40:1, nucleotide ratio, 0.4 pmol of primer termini), 0.02 pmol of Polδ or 0.03 pmol of Polδ*, and PCNA as indicated. Incubations were for 5 min at 37 °C. The assays were stopped with 7 μl of 95% formamide, 20 mM EDTA and electrophoresed on a 12% denaturing polyacrylamide gel.

Polδ Holoenzyme Assay on mp18 DNA—The standard 30-μl reaction contained 40 mM Tris-HCl, pH 7.8, 8 mM $MgAc_2$, 0.2 mg/ml bovine serum albumin, 1 mM dithiothreitol, 100 μM each of dATP, dCTP, and dGTP, and 25 μM [^{3}H]dTTP (100 cpm/pmol dNTP), 0.5 mM ATP, 40 fmol (100 ng) of singly primed SS mp18 DNA (the 36-mer primer is complementary to nt 6330–6295), 850 ng of *Escherichia coli* single stranded-binding protein, 75 mM NaCl, 100 fmol of RFC, and Polδ, Polδ*, and PCNA as indicated in the legend to the figures. Incubations were at 37 °C for the times indicated. The reactions were stopped and acid insoluble radioactivity was determined as described above. When an electrophoretic analysis of the replication products was carried out, [α-^{32}P]dTTP replaced [^{3}H]dTTP, and the assays were stopped with 10 μl of 60% glycerol, 50 mM EDTA, 1% SDS, and electrophoresed on a 1 or 1.5% alkaline-agarose gel.

RESULTS

Overexpression of the Subunits of Polδ—An inducible system for the overexpression of the Polδ genes allows normal cell growth without possible deleterious effect on cells due to constitutive high levels of the Polδ subunits. The strain used in this study is the protease-deficient strain BJ2168. In this strain, the expression of genes placed under control of the *GAL1–10* upstream activating sequence is appropriately induced by addition of galactose to the media, but the strain grows very poorly on galactose as sole carbon source. Satisfactory cell growth was obtained on media containing as carbon source 3% glycerol, 2% lactate, and a non-repressing concentration of glucose (0.1%). Galactose was added to this media to induce expression. The three Polδ genes were cloned under control of the bi-directional *GAL1–10* upstream activating sequence as described under "Materials and Methods." Constitutive overproduction of Polδ is inhibitory to yeast cell growth as a strain carrying the three overexpression plasmids grew less well on galactose medium than on raffinose, a non-inducing carbon source (data not shown).

Cells containing one or more of the Polδ genes under *GAL1–10* control were grown up to mid-logarithmic phase, induced with galactose, and crude extracts were made as described under "Materials and Methods." These extracts were assayed for DNA polymerase activity. However, because Polα constitutes the major polymerase activity in yeast extracts, a moderate overproduction of Polδ would not be readily apparent when assayed in crude extracts. Therefore, Polα activity was specifically inhibited with the dGTP analog BuPhdGTP. Under those conditions approximately one-half of the total DNA polymerase activity in crude extracts from a protease-deficient strain can be ascribed to Polδ (34).

Overexpression of the *POL3* gene alone resulted in an over 100-fold increase in *POL3* mRNA levels, a strong increase in Pol3p polypeptide levels, and an 8-fold increase in BuPhdGTP-resistant DNA polymerase activity in cleared lysates (Table I, Fig. 1, data not shown). However, this polymerase activity was unstable and most of the increased activity was lost upon further fractionation by ammonium sulfate precipitation and phosphocellulose chromatography. A Western analysis showed that most of the Pol3p polypeptide precipitated during dialysis of the redissolved ammonium sulfate pellet, and some precipitated during dialysis after phosphocellulose chromatography (data not shown). These data indicate that the catalytic polypeptide of Polδ is active but unstable. The same results were obtained when overexpression of *POL3* together with *POL32* was carried out. Overproduction of polymerase activity

TABLE I
Overproduction of Polδ

DNA polymerase assays on activated DNA were carried out in the presence of BuPhdGTP to eliminate most of the contribution due to Polα. Activities were determined in the cleared lysate (lysate), after dialysis of the redissolved ammonium sulfate precipitate ($(NH4)_2SO_4$), after dialysis following phosphocellulose chromatography (PC), and after MonoQ HPLC. Activities are relative to that of the strain lacking the overexpression plasmid.

POL genes	Relative DNA polymerase activity			
	Lysate	$(NH4)_2SO_4$	PC	MonoQ[a]
None (vector)	1	1	1	1
POL31 + *POL32*	1.2			
POL3	8	2	1.5	2.2
POL3 + *POL31*	14	12	13	17[b]
POL3 + *POL32*	9	2.5	2.2	2.6
POL3 + *POL31* + *POL32*	20	22	18	32

[a] Polδ activity only.
[b] Includes Polδ* activity.

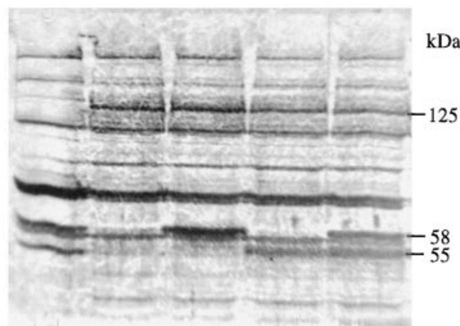

FIG. 1. **Overproduction of the subunits of Polδ.** Whole cell extracts were made of strains overexpressing, *POL31* + *POl32* (*lane 1*), *POL3* (*lane 2*), *POL3* + *POL31* (*lane 3*), *POL3* + *POL32* (*lane 4*), *POL3* + *POL31* + *POL32* (*lane 5*). The extracts were separated by 10% SDS-PAGE and subjected to Western blotting with antiserum raised to Polδ.

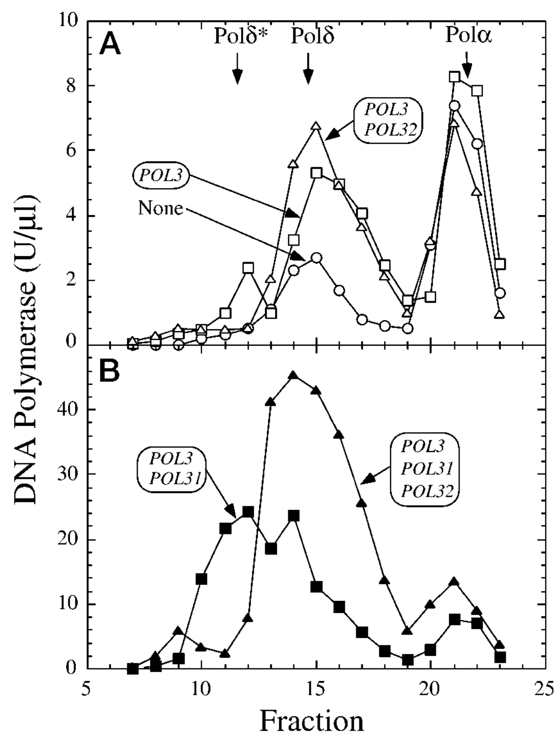

FIG. 2. **MonoQ fractionation of DNA polymerases from overproducing strains.** *A,* from a wild-type strain without an overexpression plasmid (*None*, ○) or one overexpressing *POL3* (□) or *POL3* + *POL32* (Δ). *B,* from a strain overexpressing *POL3* + *POL31* (■) or *POL3* + *POL31* + *POL32* (▲). Note the difference in the *y* axis scale between *panels A* and *B.*

in the cleared lysates was 9-fold, but the activity also decayed upon further fractionation (Table I). Again, by Western analysis, Pol3p was found in the insoluble pellet whereas most of the overproduced Pol32p remained soluble. A gel filtration analysis of the enzyme fraction after phosphocellulose chromatography failed to identify an activity which contained both Pol3p and Pol32p but lacked Pol31p (data not shown). These data suggest that Pol3p does not form a stable active complex with Pol32p.

In contrast, overexpression of *POL3* together with *POL31,* or of *POL3* together with *POL31* and *POL32* resulted in a much larger increase in polymerase activity in crude extracts (Table I). Yet, the polypeptide levels in these extracts were not higher than when Pol3p was overproduced alone or together with Pol32p (Fig. 1). In addition, the polymerase activity from these strains remained stable during ammonium sulfate fractionation, phosphocellulose chromatography, and MonoQ HPLC (Table I). Overexpression of *POL31* together with *POL32* gave an increase in the levels of these subunits, but no significant increase in DNA polymerase activity, indicating that the level of Pol3p limits the level of Polδ in the cell (Fig. 1, Table I).

The partially purified preparations were fractionated on a strong anion exchanger (MonoQ), which separates Polδ from Polα and Polϵ. In comparison to the control strain a 2.2-fold increase in Polδ activity was measured in the MonoQ fractions when Pol3p alone was overproduced, and a 2.6-fold increase when Pol3p and Pol32p were overproduced together (Fig. 2*A* and Table I). Interestingly, two poorly separated peaks of DNA polymerase activity resulted from overproduction of Pol3p together with Pol31p. Whereas the elution position of the minor peak coincides with that of Polδ, the earlier eluting major peak represents a novel activity (Fig. 2*B*). The elution position is similar to that observed during fractionation of extracts from a mutant strain lacking Pol32p (7). To show that this novel peak represents a two-subunit form of Polδ, we overproduced Pol3p together with Pol31p in a protease-deficient *pol32*Δ strain. Only the early eluting peak, and no Polδ peak, was observed, and this activity was increased more than 10-fold in fractions from the overproducing strain (Fig. 3). For the purpose of this study we identify the two-subunit form of Polδ as Polδ*.

Both Polδ and Polδ* were further purified by chromatography on a strong cation exchanger. Interestingly, Polδ* again eluted prior to Polδ, indicating that the highly charged nature of Pol32p promotes retention of Polδ on either ion-exchange matrix. After the MonoS step, Polδ was about 95% pure and Polδ* 50% pure (data not shown). They were further purified by gel permeation chromatography as described below.

*Subunit Structure of Polδ and Polδ**—Enzyme from the MonoS step was injected on a Superose 6 column. Polδ eluted at a position consistent with that of a 520-kDa complex (Fig. 4). This is in agreement with our initial studies of Polδ in which we noted that the apparent size of Polδ was >300 kDa (11). In contrast, the elution position of Polδ* indicated a size of 180 kDa for that complex (Fig. 4). The data shown in Fig. 4 were obtained when concentrated enzyme at 0.4 mg/ml was injected onto the column. Dilution of the injected enzyme to 0.05 mg/ml did not change the respective elution positions of Polδ and Polδ* (data not shown).

To obtain an estimate of the subunit stoichiometry, the peak activity fractions of the Superose 6 column were analyzed on a 10% SDS-polyacrylamide gel. The proteins were stained with Coomassie Brilliant Blue and the stained gels were digitized and quantitated. Scanning results indicate a stoichiometry for

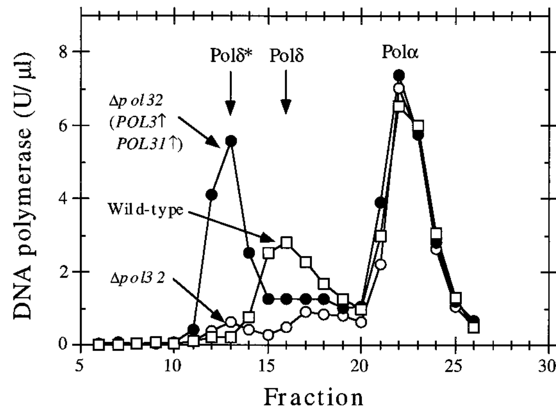

FIG. 3. **MonoQ fractionation of DNA polymerases from overproducing strains lacking Pol32p.** The profiles from a wild-type strain (□), from the isogenic *pol32*Δ mutant (○) and from the mutant strain overexpressing *POL3* + *POL31*.(●, shown with ↑) are shown.

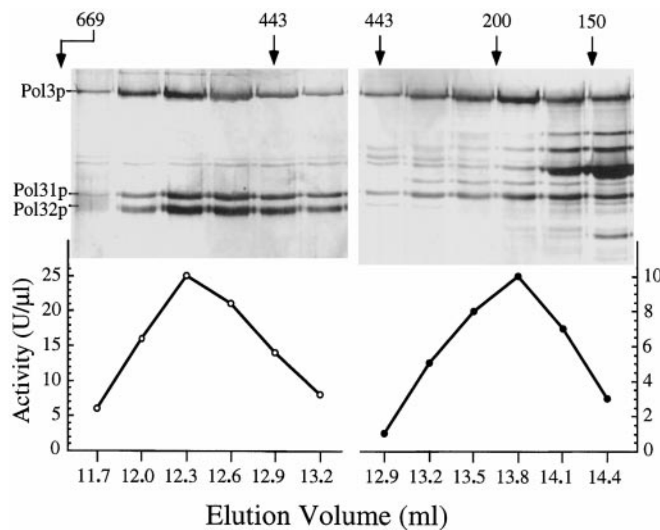

FIG. 4. **Subunit structure of Polδ and Polδ*.** The peak MonoS fraction of Polδ (*left panel*) or Polδ* (*right panel*) was analyzed on a Superose 6 column. DNA polymerase activity of the fractions was measured on activated DNA. Proteins were separated by 10% SDS-PAGE and visualized by silver staining. Size markers are thyroglobulin (669 kDa), apoferritin (445 kDa), β-amylase (200 kDa), and yeast alcohol dehydrogenase (150 kDa). The elution volume of thyroglobulin was 11.2 ml.

Polδ* of Pol3p:Pol31p = 1.0:0.9, and for Polδ of Pol3p:Pol31p:Pol32p = 1.0:1.05:1.1 (Fig. 5). Together with the gel filtration results, these data give as the most likely structure for Polδ* that of a heterodimer (Pol3p·Pol31p), and for Polδ that of a hexamer: a dimer of a heterotrimer $(\text{Pol3p·Pol31p·Pol32p})_2$.

*Enzymatic Activities of Polδ and Polδ**—The specific activity of Polδ and Polδ* was measured on activated DNA (data not shown). Calculated on a weight basis Polδ had a 1.3-fold higher specific activity than Polδ*. This equates to a 3.2-fold higher specific activity for Polδ if calculated on a molar basis, or 1.6-fold higher if both catalytic cores in the hexameric Polδ are active.

The processivity of both enzymes was determined on $\text{poly(dA)}_{500}\cdot\text{oligo(dT)}_{22}$. Whereas Polδ* incorporated 2–3 dTMP residues per binding event, Polδ had a processivity of 6–12, in agreement with previous studies (Fig. 6 and Ref. 12). Upon addition of PCNA to the assay, both enzymes were fully processive. However, a much higher level of PCNA was necessary to stimulate processive DNA synthesis by Polδ* than by Polδ (Fig. 6).

Polδ Poorly Replicates Natural DNA Templates*—DNA synthesis by Polδ* or Polδ on extended SS DNA templates is virtually completely inhibited by the presence of 75 mM NaCl in the assay. On the other hand, these conditions are optimal for replication by a complex of Polδ with PCNA (13). PCNA was loaded onto singly primed single stranded-binding protein-coated SS mp18 DNA by RFC and ATP. Addition of Polδ resulted in extremely fast and efficient DNA synthesis (Fig. 7*A*).

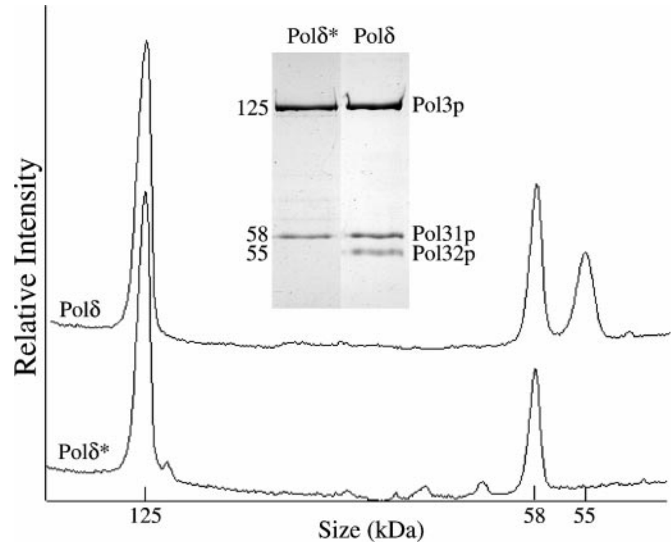

FIG. 5. **Subunit stoichiometry of Polδ and Polδ*.** Peak fractions from the gel filtration column (the 12.3-ml fraction of Polδ and the 13.5-ml fraction of Polδ*, Fig. 4) were separated by 10% SDS-PAGE and the gel stained with Coomassie. The gel was digitized with a CCD camera and lanes scanned and quantitated using the NIH Image software. The tracings are shown as well as a contrast-enhanced view of the actual gel used for quantitation. The molar ratio of subunits was, for Polδ*:Pol3p:Pol31p = 1:0.9; and for Polδ, Pol3p:Pol31p:Pol32p = 1.0:1.05:1.1.

FIG. 6. **Replication of $\text{poly(dA)}\cdot(\text{dT})_{22}$ by Polδ* and Polδ.** Reactions contained no PCNA (*lanes 1* and *5*), 0.06 pmol (*lanes 2 and* 6), 0.6 pmol (*lanes 3* and *7*), or 6 pmol of PCNA (*lanes 4* and *8*). The length of the poly(dA) is about 500 nt. See "Materials and Methods" for further details.

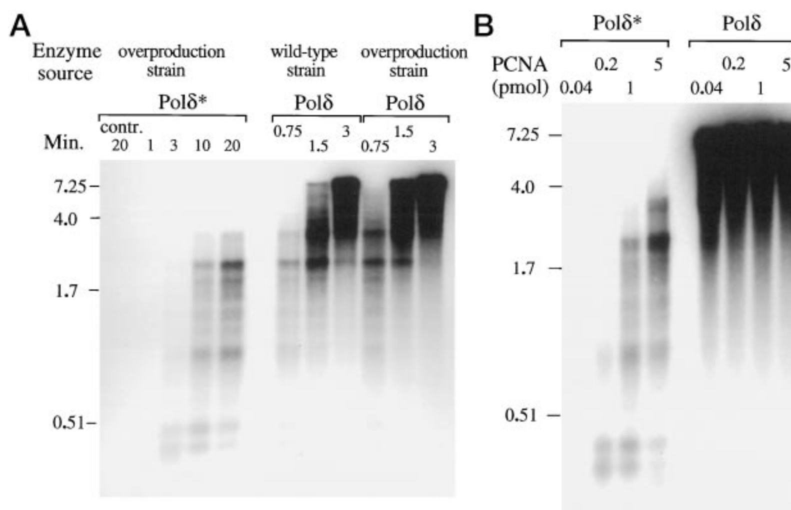

FIG. 7. **Replication of SS mp18 DNA by Polδ* and Polδ.** *A,* the 60-μl holoenzyme replication reactions contained 0.08 pmol of primed template, 0.2 pmol of RFC, 1 pmol of PCNA, and either 0.2 pmol of Polδ* or 0.1 pmol (expressed as hexamers) of Polδ (see "Materials and Methods" for details). Aliquots of 15 μl were analyzed on a 1.5% alkaline-agarose gel. The control lane (*lane 1*) is from a reaction with Polδ* lacking RFC. The two different forms of Polδ as indicated are the ones purified from wild-type strain BJ405 (7), and from the overproducing strain (Fig. 4). Size markers (in kb) are indicated at the *left*. *B,* standard replication assays contained 0.04 pmol of primed template, either 0.1 pmol of Polδ* or 0.05 pmol (expressed as hexamers) of Polδ and PCNA as indicated. Assays were for 5 min at 37 °C and were analyzed on a 1.5% alkaline-agarose gel. Size markers are in kb.

Only marginal differences in replication efficiency were observed between Polδ purified from a wild-type strain of yeast through a six-column procedure and Polδ purified from the overproducing strain through a three-column procedure. The fastest complexes complete replication of the 7,250-nt mp18 circle within 1.5 min at 37 °C, a rate of more than 80 nt/s. In comparison, Polδ* is a very inefficient enzyme, replicating only ~2 kb of DNA during the 20-min assay (Fig. 7*A*). Replication is still dependent on PCNA as no synthesis was observed in its absence. Pause sites with Polδ* are much more pronounced than with Polδ, indicating that sites of secondary structure form major replication barriers for this two-subunit enzyme (Fig. 7).

The poor replication efficiency of Polδ* may be due to the frequent disassembly of replication complexes, perhaps at sites of secondary structure. In that case, rapid reassembly should be stimulated by providing excess PCNA or excess Polδ* resulting in more efficient synthesis. Indeed, high levels of PCNA stimulated Polδ*-mediated replication (Fig. 7*B*). In comparison, because Polδ holoenzyme is a very stable complex, a slight molar excess of PCNA over primer termini already allowed maximally efficient synthesis by Polδ (Fig. 7*B*). Similarly, a 10-fold molar excess of Polδ* over primer termini also greatly stimulated PCNA-dependent replication of SS mp18 DNA by this enzyme (data not shown).

Reconstitution of Polδ from Polδ with Pol32p*—Pol32p overproduced in *E. coli* is found in inclusion bodies. The protein was efficiently renatured from a 6 M urea extract. Its properties indicate that Pol32p is a homodimer (7). Polδ* was incubated with renatured *E. coli* expressed Pol32p, and assayed in a holoenzyme assay on single-stranded mp18 DNA, in order to determine whether *in vitro* reconstitution of the three-subunit Polδ could be performed. The result in Fig. 8*A* shows that reconstitution of Polδ as measured by the formation of a processive holoenzyme proceeded quite efficiently. Controls, a dialyzed urea extract from *E. coli* cells or from cells overproducing Pol31p, show that reconstitution is specific for Pol32p.

To determine whether Pol32p would restore the dimeric structure of Polδ upon reconstitution, the *E. coli* produced subunit was purified to ~60% homogeneity by MonoQ HPLC, preincubated with an equimolar quantity of Polδ*, and subjected to Superose 6 gel filtration. DNA polymerase activity, as measured on activated DNA, eluted as two poorly separated peaks at ~500 and ~200 kDa (Fig. 8, *B* and *C*). When the fractions were assayed for activity in a Polδ holoenzyme assay on SS mp18 DNA, the low molecular weight peak showed up as a mere shoulder to the high molecular weight peak (Fig. 8*B*). An SDS-PAGE analysis showed that the peak at 500 kDa contained all three subunits of Polδ including Pol32p (Fig. 8*C*). The peak at 200 kDa also contained low amounts of Pol32p, but considering its low replication activity on SS mp18 DNA, functional Polδ was likely not reconsituted.

DISCUSSION

Overproduction Studies in Yeast—Overproduction of Polδ in yeast was easily accomplished by cloning the genes for its three subunits under control of the galactose-inducible *GAL1–10* promoter (Table I). The enzyme isolated and purified from such an overproduction strain did not show marked differences with the enzyme isolated from a wild-type strain (Fig. 7). The slightly lower activity of Polδ isolated from a non-overproducing strain could be caused by partial inactivation of the enzyme during the laborious multistep purification procedure. (7). The availability of overproducing plasmid also allowed us to investigate the occurrence of partial Polδ complexes and their activities. A yeast two-hybrid analysis with Polδ subunits indicated a strong interaction between Pol3p and Pol31p and between Pol31p and Pol32p, and also a weak but significant interaction between Pol3p and Pol32p (7). Yet, we found no biochemical evidence for a stable complex between Pol3p and Pol32p. When both subunits were overproduced together and the extracts fractionated on a MonoQ column, the same results were obtained as when only Pol3p was overproduced: only one peak of Pol3p derived activity was identified, and this peak corresponded to the normal elution position of Polδ (Fig. 2A). Although these biochemical data do not exclude the possibility of a Pol3p·Pol32p complex as suggested by the two-hybrid analysis, they do indicate that such a complex would be as unstable as the catalytic subunit alone. An alternative explanation of the two-hybrid results is that the Pol3p·Pol32p signal was the result of an indirect interaction with Pol31p serving as a bridge, *i.e.* Pol3p·Pol31p·Pol32p. Pol3p forms a strong complex with Pol31p to give Polδ*, and Pol31p also forms a strong complex with Pol32p, as do the analogous subunits in *S. pombe* (6, 7, 14).

Overproduction of the catalytic subunit alone or together with Pol32p resulted in a 2–3-fold overproduction of Polδ from the MonoQ column (Fig. 2*A*). The most simple explanation of these results is that in wild-type yeast cells, Pol31p and Pol32p are present at higher levels than Pol3p, and that, therefore, overexpression of Pol3p alone or together with Pol32p is sufficient for the observed moderate overproduction of Polδ. In agreement with this conclusion is the observation that over-

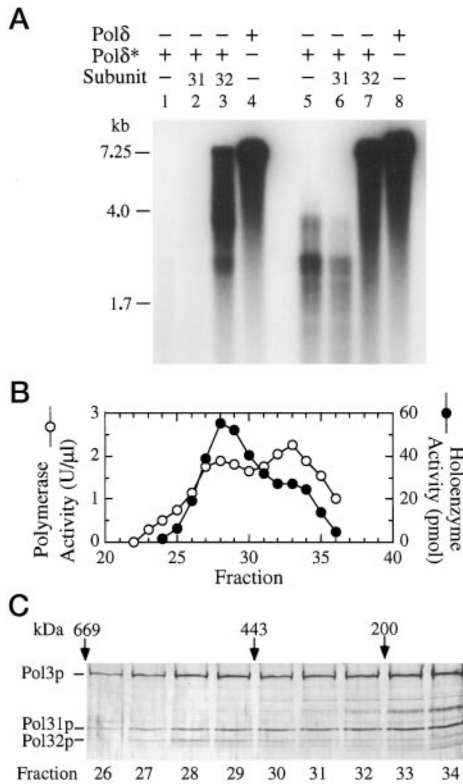

FIG. 8. **Reconstitution of Polδ from Polδ* and Pol32p.** *A,* cell pellets from lysed *E. coli* cells or from cells overexpressing *POL31* or *POL32* were extracted with a buffer containing 6 M urea, and the extract was dialyzed stepwise to a no urea buffer (7). Polδ* (0.5 μg) was incubated with 5 μl of dialysate, which contained either no overproduced protein (*lanes 1* and *5*) or approximately 1 μg of Pol31p (*lanes 2* and *6*) or 2 μg of Pol32p (*lanes 3* and *7*), in a total volume of 20 μl of 40 mM Hepes-NaOH, pH 7.5, 10% glycerol, 1 mM EDTA, 0.02% Nonidet P-40, 0.2 M NaCl, 5 mM DTT, 5 mM $NaHSO_3$, and 2 μM each of leupeptin and pepstatin A. After an incubation for 1 h on ice, standard mp18 DNA replication assays were carried out in an assay containing 0.5 pmol of PCNA, 2 μl (0.2 pmol) each of the reconstituted enzymes (*lanes 1–3* and *5–7*), or 0.05 pmol (expressed as hexamers) of Polδ (*lanes 4* and *8*). Aliquots taken after 2 min (*lanes 1–4*) and 10 min (*lanes 5–8*) were analyzed on a 1.5% alkaline-agarose gel. *B,* the dialyzed Pol32p obtained from a 1-liter *E. coli* overproduction scale was further purified by MonoQ HPLC in buffer C using a NaCl gradient from 100 to 500 mM. The subunit eluted broadly between 200 and 350 mM NaCl. The purest fraction (~60% pure) eluting at 300 mM NaCl (100 μl, 12 μg of protein representing ~7 μg = ~0.18 nmol monomer of Pol32p) was incubated with 50 μl (23 μg = 0.13 nmol of heterodimer) of Polδ* for 1 h at 0 °C and analyzed on a Superose 6 column. Fractions of 0.3 ml were collected and analyzed for DNA polymerase activity on activated DNA (○) and for holoenzyme activity on mp18 DNA (●). *C,* proteins in the peak fractions were separated by 10% SDS-PAGE and visualized by silver staining. Fractions 26, 30, and 34 represent elution volumes of 11.8, 12.8, and 13.8 ml, respectively. The elution positions of markers is as indicated. For details see "Materials and Methods" and the legend to Fig. 4.

production of Pol31p and Pol32p did not result in increased polymerase activity (Table I).

Properties of Polδ and Polδ*—Most interestingly, a novel complex, Polδ*, was isolated from the simultaneous overproduction of Pol3p and Pol31p. The differences between Polδ* and Polδ are both structural and enzymatic. (i) Polδ* is a heterodimer, whereas Polδ is a dimer of a heterotrimer (Figs. 4 and 5). With the knowledge that Pol32p by itself forms a homodimer, it follows that dimerization of the catalytic core must be the result of Pol32p dimerization (7). In fact, addition of Pol32p to Polδ* restored the dimeric form of Polδ (Fig. 8, *B* and *C*). (ii) The processivity of Polδ alone on poly(dA)·oligo(dT) is higher than that of Polδ* (Fig. 6). (iii) In the presence of PCNA both DNA polymerases are fully processive on poly(dA)·oligo(dT). However, much more PCNA is required to make processive complexes with Polδ* than with Polδ (Fig. 6). PCNA rapidly dissociates from linear DNA and is only stabilized onto the DNA by interaction with the polymerase (15–17). Therefore, PCNA trimers loaded by diffusion onto poly(dA)·oligo(dT) rapidly slide off the DNA as they fail to be anchored much less efficiently by Polδ* than by Polδ. However, those few PCNA clamps which do form a complex with Polδ*, replicate processively. (iv) Polδ* holoenzyme (*i.e.* the complex of polymerase, PCNA, and RFC) is much less efficient than Polδ holoenzyme in the replication of natural DNA templates (Fig. 7). Whereas Polδ holoenzyme replicated SS mp18 DNA with high processivity, replication by Polδ* holoenzyme showed frequent pausing. Those replication defects were partially suppressed by a large molar excess of PCNA or Polδ*, suggesting that pausing led to frequent holoenzyme disassembly, and subsequent reassembly was stimulated by excess PCNA and Polδ* (Fig. 7*B*, data not shown). As the PCNA·Polδ* complex is processive on poly(dA)·oligo(dT) which lacks secondary structure, it is likely that the replication defects of Polδ* holoenzyme on SS mp18 DNA are due to the extensive secondary structure of this template. In conclusion, although the interactions between PCNA and the Pol3p and/or Pol31p subunits are essential for establishing a productive PCNA-polymerase complex, additional interactions between PCNA and Pol32p may stabilize this complex, particularly during replication of secondary structures in the DNA template. Alternatively, or in addition, the presence of the third subunit itself may stabilize the holoenzyme complex.

Yeast Polδ Is Comparable to Human Polδ*—The two-subunit yeast Polδ* is structurally and functionally analogous to mammalian Polδ (1, 18). Like Polδ*, mammalian Polδ is purified as a heterodimer. Depending on the PCNA levels, the synthetic rate of Polδ* holoenzyme varies from 1.5 to 15 nt/s, much less than the rate of the three-subunit Polδ at ~100 nt/s (Fig. 7) (19). The latter rates are comparable to *in vivo* rates of fork movement in yeast (20, 21). The range of synthetic rates of human or bovine Polδ holoenzyme in analogous replication reactions is 2–10 nt/s (*e.g.* see Refs. 22 and 23). As with yeast Polδ* holoenzyme, replication by mammalian Polδ holoenzyme is also prone to pausing. It appears that the large difference in replication efficiency between yeast and mammalian Polδ is largely or completely accounted for by the presence of the third subunit in the yeast enzyme. Is this subunit also present in mammals? As Pol32p and Cdc27, the functional *S. pombe* homologue of Pol32p, show only minimal sequence similarity, it may not be possible to clone or identify this putative mammalian subunit based on sequence comparison considerations. The prediction would be that human Polδ containing the third subunit would be more processive. SV40 might be an attractive assay system for this third subunit as the assay can be carried out in crude extracts (24). Unfortunately, as the synthetic rate of the SV40 fork is limited at 3 nt/s by the rate of the T antigen helicase, it may not be possible to functionally detect the presence of the third subunit, based on rate considerations only (22).

Is Polδ a Dimer at the Fork?—The observation that Polδ has a dimeric catalytic core immediately suggests the notion that this enzyme is also a functional dimer at the replication fork. Replication of both the leading and the lagging strand by Polδ is the favored model for SV40 (25, 26). However, in yeast there are several indications that Polε plays a major role at the replication fork. First, Polε is essential for yeast cell growth and the phenotypes of temperature-sensitive Polε mutants indicate that the enzyme is required for the elongation phase of DNA replication (27, 28). Second, *in vivo* cross-linking studies place Polε at or near the fork (29). Third, the mutator pheno-

type of a proofreading-deficient mutant of Polϵ suggests a replication function for Polϵ. In particular, the multiplicative relationship of spontaneous mutation rates between Polϵ exonuclease-deficient mutants and mismatch repair mutants indicates that these pathways act sequentially, *i.e.* that the proofreading function of Polϵ is required during DNA replication and prior to mismatch repair (30). Furthermore, the observed spectrum of 6-*N*-hydroxylaminopurine-induced mutations in strains defective for the proofreading exonuclease of either Polδ or Polϵ indicate that the respective exonuclease functions of these DNA polymerases correct the analog induced DNA replication errors on opposite DNA strands (31). By extension, if we assume that the DNA synthetic and proofreading functions of a DNA polymerase are tightly coupled, these latter data indicate that Polδ and Polϵ replicate opposite strands of the fork (32). Taken in total, the various data pointing to the respective replication functions of these two DNA polymerases remain inconclusive and perhaps even contradictory.

Function of Pol32p—The Pol32p subunit of Polδ has at least three functional domains, a basic structural domain for interaction with Pol31p, an organizational domain for homodimer formation thereby promoting dimerization of the catalytic core, and a domain which interacts with PCNA (7). None of these functions is essential for yeast cell growth. Recently, we described the properties of a PCNA mutant, pcna-79, with mutations in conserved residues in the interdomain connector loop (I126A, L128A) (33). The mutant PCNA fails to interact with the Pol32p subunit and the *in vitro* replication properties of a pcna-79 containing Polδ holoenzyme are quite similar to those of the Polδ* holoenzyme described here, suggesting that the observed *in vitro* phenotype of Polδ* holoenzyme may be due to the loss of a PCNA interaction site. However, mutant yeast cells containing the *pol30-79* mutation differ in phenotype from mutants deleted for the *POL32* gene. Whereas both mutants are sensitive to hydroxyurea indicative of replication defects, *pol32*Δ mutants are cold-sensitive for growth but *pol30-79* mutants are not. Furthermore, *pol30-79* is a mutator and *pol32*Δ an antimutator with a defect in damage-induced mutagenesis (7, 33). Loss of Pol32p function shows a much more severe growth defect than loss of the PCNA-Pol32p interaction. Possibly, the loss of dimerization function of Pol32p rather than PCNA interaction leads to the observed conditional lethal phenotype in *pol32*Δ mutants. If the function of Polδ as a dimeric enzyme is important for yeast replication, it appears that other factors contribute to the stabilization of a dimeric replisome, and, therefore, loss of Pol32p-Pol32p interactions can be tolerated. A comprehensive mutational analysis of the *POL32* gene is required to address these questions appropriately.

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